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14. ABSTRACT

This proposal was initially based on the IDEA award concept that the abnormal gene promoter DNA methylation status of the AP2 gene might predict which DCIS lesions in women would be at risk for the evolution of recurrence and/or emergence of invasive cancer. As the work progressed the concept was expanded to include the DNA methylation status of additional genes for this purpose and also for the purpose of predicting survival outcomes in women with all stages of breast cancer. During the course of the project, we performed a small nested case control study of 71 women with DCIS from USCF of which 34 developed recurrent disease. Unfortunately, among the methylation status of the AP2, CYCLIN D, ECAD, GSTP, and SSOCS genes, either as individual genes, or in combinations, no significant odds ratios for disease occurrence emerged when the predictive value of nuclear grade was factored in. We also performed a large blinded study of over 140 women with DCIS and all stages of breast cancer in a cohort from New Mexico with extensive 5 year longitudinal follow-up. Unfortunately, for all of the above genes plus 5 others, multivariate analyses, to date, do not show significant OR's for recurrence/survival.

15. SUBJECT TERMS
Breast Cancer, DCIS, *AP-2α,* Hypermethylation, SOCSI, *E-cad* genes

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Introduction

The overall purpose of this Idea Award was, as stated in the originally funded proposal, **t*o determine whether hypermethylation of the AP-2α promoter and of other promoters where the marker incidence looks promising, could serve as molecular markers to predict the potential for breast DCIS (ductal carcinoma in-situ) lesions from individual patients to recur as invasive breast cancer***. In-situ carcinomas account for approximately 20% of all newly diagnosed breast cancers in women. So the question of predicting which of these lesions which may progress, following initial surgery, to invasive breast cancers is an extremely important one especially since this may occur in some 15 to 20% of women diagnosed with DCIS. For our objective, we are employing a newly established, and highly sensitive, PCR assay for hypermethylation of *AP-2α* exon1, and for other candidate genes, which can be utilized on DNA scraped from slides prepared from paraffin blocks. In carrying out our overall objective, our study has three specific aims which have remained totally unchanged from the original proposal and these are:

Task/Specific Aim #1 – To verify the low incidence of *AP-2α* exon 1 hypermethylation in pure DCIS and compare this incidence in a progression model that assesses methylation in DCIS adjacent to and in the invasive cancer.

Task/Specific Aim #2 – To conduct a nested case control study, in collaboration with the UCSF Breast Cancer SPORE group, of *AP-2α* hypermethylation in DCIS samples from patients who have and have not manifested recurrent invasive disease.

Task/Specific Aim #3 – To conduct a prospective study of the predictive power for *AP-2α* hypermethylation in a cohort of women from the state of New Mexico with DCIS.

Body

During this award, we developed a panel of promoter DNA hypermethylated genes which, collectively, appeared to hold promise for predicting the behavior of DCIS. We utilized these markers during the grant to perform the following specific aims:

Specific Aims #1 and 2:

A. *Work during the initial funding period*: Over the cost of the regular funding period, we studied our gene panel for its predictive value in 70 extremely well annotated and followed patients from UCSF who had and had not gone on to recurrent disease and found performance of the individual genes is shown in **Table 1. Cyclin D and GSTPi together provided a 4.0 odds ratio (not shown) for recurrence but this was not as high as nuclear grade, the best clinical parameter, done on these same tumors from the patients.**

Table 1. Univariate results of estimate of risk of DCIS recurrence associated with methylation of select genetic markers and nuclear grade (N=71).

Variable*	Recurrent DCIS % (N=34)	Non-Recurrent DCIS % (N=37)	Odds Ratio (95% Confidence Interval)	P+
AP2				
M§	12	5	2.27 (0.39 to 13.27)	0.36
UM	88	95	1.00 (referent)	
CYCLIN D				
M	47	36	1.57 (0.61 to 4.10)	0.35
UM	53	64	1.00 (referent)	
ECAD				
M	0	3		
UM	100	97		
GSTP				
M	40	24	2.02 (0.72 to 5.64)	0.19
UM	60	76	1.00 (referent)	
SSOCS				
M	50	42	1.36 (0.51 to 3.61)	0.54
UM	50	58	1.00 (referent)	
Nuclear grade				
High	64	25	6.42 (1.61 to 25.64)	<0.01
Intermediate	24	44	1.38 (0.33 to 5.72)	0.66
Low	12	31	1.00 (referent)	

*Missing data: 1.4% for AP2, CYCLIN D, ECAD, GSTP; 4.2% for SSOCS; 2.8% for nuclear grade.
§M=methylated; UM=unmethylated;
+Two-sided; calculated based on χ^2 test;

However, there was not a strict correlation between the two parameters and actually, when a multivariate analysis was done taking the hypermethylated genes and the nuclear grade parameter together (**Table 2**), the hypermethylated genes still give an over 3.0 OR for disease recurrence.

Table2. Multivariate results of estimates of risk of DCIS recurrence from final logistic regression model of genetic markers and nuclear grade.

Variable	Odds Ratio (95% Confidence Interval)	P
Nuclear Grade		
High	6.00 (1.46 to 24.71)	0.01
Intermediate	1.37 (0.31to 6.08)	0.67
Low	1.00 (referent)	
CYCLIN D & GSTP		
M	**3.30 (0.73 to 15.04)**	**0.12**
UM	**1.00 (referent)**	

These results are continuing to be analyzed by the UCSF group and it seems apparent that hypermethylated genes are picking up a group of patients that even nuclear grade does not. We believe

another ~ 40 patients (half of who did and half who did not recur) will give us a final look at the results. We are encouraged that these will suggest that development of a panel of DNA hypermethylated genes can truly help evolve a better management of patients with DCIS.

Final no cost extension year: ***The UCSF group considered all of the above analyses and concluded that the heavy contribution of nuclear grade made it unlikely that further sample analyses from their cohort, considering the precious nature of the small samples, would not be helpful. We thus turned to more DCIS samples from the New Mexico group, outlined in the next specific aim for further data concerning DCIS and the results are outlined in the aim below.***

Specific Aim #3

A. *Work during the initial funding period*: To conduct a prospective study of the predictive power for *AP-2α* hypermethylation in a cohort of women from New Mexico with DCIS. This was the other ultimate study in the proposal utilizing the cohort from Dr. Belinsky in which DCIS samples, over 100, come from a 3,000 patient study of women with early stage breast cancer and/or DCIS in New Mexico. These patients have complete 5 year longitudinal follow-up and this has just been successfully complied into a finalized database. **We now have actually studied all of the samples from all of the women with DCIS that have been identified.** In addition to examining the methylation status of *AP-2α* , we have expanded the work to look at each of the other candidate genes in our panel discussed in detail in Specific Aims #1 and 2. The longitudinal data for recurrence are now in progress by Dr. Belinsky and his colleagues and we will match the results to our findings as soon as these are available. We now anticipate this match within the next four to six weeks.

Final no cost extension year: We are still conducting assays and data analysis in the cohort but have broken the code on the numbers of samples we were able to process to date in the New Mexico cohort. First, we are addressing the question of women with DCIS to determine if we could further validate any trends seen in the UCSF study conducted in Specific Aim # 1. The data for AP2, alone are shown in Table 3, and data from additional samples and the other genes are still pending at this time including evaluation for performance of groups of markers together. Unfortunately, the AP2 data do not show a positive correlation with recurrence, to date, as shown below.

Table 3: DCIS Recurrence Study
Results for AP2, restricted to women with insitu breast cancer

Gene	Frequency* (%)		Adjusted for design (age)		Adjusted for design (age) & ethnicity	
	Recurred (n=8)	Without recurrence (n=14)	Odds Ratios (95% CI)	p-value	Odds Ratios (95% CI)	p-value
Ap2	25.0	28.6	0.24 (0.01, 7.73)	0.73	0.30 (0.01, 7.29)	0.76

*Samples included in study and for which AP2 results were obtained.

We have also done an analysis of AP2 results across all women analyzed in the cohort with all stages of breast cancer (Table 4). Although there are positive trends for prediction

Table 4: Stage Distribution Study: Results for AP2

Frequency*			Unadjusted				Adjusted for age & ethnicity		
			Odds ratios (95% CI)		p-value		Odds ratios (95% CI)		p-value (exact)
Stage			Comparison to Local Stage				Comparison to Local Stage		
In-situ (n=22)	Local (n=42)	Regional (n=31)	In-situ	Regional	Exact	trend	In-situ	Regional	
40.9	23.8	35.5	2.22 (0.73, 6.70)	1.76 (0.63, 4.89)	0.34	0.80	2.34 (0.72, 7.62)	1.38 (0.46, 4.15)	0.37

*Samples included in study and for which ap2 results were obtained.

We are also continuing analyses for the multiple markers performed in the New Mexico data for women with all stages of breast cancer and data for individual genes have just been done (Patient characteristics, Table 5; individual gene results, Table 6).

Table 5 Characteristics of Study Participants: Recurrence Study

Characteristic		Status at 5 years	
		Recurred	No recurrance
N (%)		74 (51.7)	69 (48.3)
Stage (%)	In-situ	8 (10.8)	16 (23.2)
	Local	42 (56.8)	47 (68.1)
	Regional nodes	24 (32.4)	6 (8.7)
Age at diagnosis (%)	[35,50)	25 (33.8)	24 (34.8)
	[50,65)	25 (33.8)	24 (34.8)
	≥ 65	24 (32.4)	21 (30.4)
Ethnicity (% Hispanic)		23 (31.1)	15 (21.7)
BMI (%)	Normal	30 (44.8)	34 (54.0)
	Overweight	26 (38.8)	17 (27.0)
	Obese	11 (16.4)	12 (19.0)
Menopausal status (% post menopause)		47 (63.5)	43 (62.3)
Immunohisto-chemistry (% positive)	P53	24 (33.3)	9 (13.4)
	Her-2/neu	15 (20.8)	16 (23.5)
Immunohisto-chemistry (% negative)	ER	23 (32.4)	11 (16.2)
	PR	35 (49.3)	19 (27.9)
Triple negative		7 (10.1)	4 (5.9)

Note: some missing data for age, BMI and the IHC variables.

Table 6 Recurrence Study: Methylation Results

Gene	Frequency (%)[#]		Adjusted for design (age)		Adjusted for design (age), stage & ethnicity	
	Recurred (n=74)	Without recurrence (n=69)	Odds Ratios (95% CI)	p-value	Odds Ratios (95% CI)	p-value
P16	30.6	30.2	1.07 (0.48, 2.40)	1.00	1.07 (0.46, 2.49)	1.00
RASSF1A	51.4	45.2	1.26 (0.60, 2.68)	0.63	1.18 (0.54, 2.64)	0.78
Dapk	**22.5**	**19.0**	**1.25 (0.50, 3.20)**	**0.75**	**1.33 (0.50, 3.68)**	**0.69**
Gstpi	29.7	26.8	1.05 (0.43, 2.59)	1.00	1.04 (0.40, 2.69)	1.00
Pax5α	30.8	28.6	1.24 (0.51, 3.08)	0.76	0.98 (0.38, 2.56)	1.00
Pax5β	30.9	33.3	0.92 (0.40, 2.13)	0.98	0.69 (0.26, 1.77)	0.53
Hcad	**16.4**	**8.9**	**1.96 (0.58, 7.67)**	**0.35**	**1.33 (0.36, 5.54)**	**0.87**
Gata5	41.4	36.2	1.13 (0.52, 2.48)	0.87	1.02 (0.44, 2.36)	1.00
cyclind	**28.2**	**16.9**	**1.85 (0.76, 4.71)**	**0.20**	**1.23 (0.45, 3.37)**	**0.82**
Ap2	12.7	21.9	0.49 (0.16, 1.39)	0.21	0.46 (0.14, 1.38)	0.20
ecad	**48.6**	**32.8**	**1.84 (0.88, 3.94)**	**0.12**	**1.62 (0.73, 3.63)**	**0.27**

[#]Results missing for 7 to 23 specimens, depending on biomarker.

As can be seen, there are positive ratios for multiple genes (DapK, H-cad,cyclinD, and E-cad) which persist after the results have been adjusted for stage, although statistical significance foe each gene alone has not been reached. We have also been analyzing the data for stage 1 disease alone (Table 7).

Table 7 Recurrence Study: Restricted to participants with local stage (stage 1)

Gene	Frequency (%)[#]		Adjusted for design (age)	
	Recurred (n=42)	Without recurrence (n=47)	Odds Ratios (95% CI)	p-value
P16	30.0	26.2	1.10 (0.37, 3.36)	1.00
RASSF1A	57.9	41.5	2.26 (0.77, 6.97)	0.15
Dapk	25.6	28.6	0.74 (0.24, 2.28)	0.73
Gstpi	31.4	32.4	0.88 (0.24, 3.13)	1.00
Pax5α	26.8	27.0	1.20 (0.36, 4.13)	0.96
Pax5β	27.0	37.8	0.47 (0.14, 1.54)	0.26
Hcad	**15.8**	**11.1**	**1.91 (0.34, 13.66)**	**0.64**
Gata5	**44.7**	**30.0**	**1.66 (0.57, 5.05)**	**0.43**
cyclind	**25.0**	**17.8**	**1.60 (0.47, 5.66)**	**0.56**
Ap2	7.5	20.4	0.26 (0.04, 1.39)	0.14
ecad	**48.7**	**36.4**	**1.99 (0.71, 5.88)**	**0.22**

Again, there are some important individual trends which could prove important but are not yet at statistical significance (note results for the GATA5, cyclinD, and especially the H-cad and E-cad genes). We are now looking again at multivariate analyses for impact of other factors and, importantly, the results of gene combinations. We are also continuing to do sample analyses.

Key Research Accomplishments

As outlined above, during the no cost extension period we have been able to add quite a bit of data. While we have not yet yielded as robust a result as we would have wished, especially for the question of recurrence prediction for DCIS, we have continued to move forwards for the question of recurrence prediction and especially for early stage disease. We have not lost enthusiasm that a DNA methylation marker approach for molecular staging of breast cancer can be achieved. We should have a clearer idea as sample analyses continue and gene combination analyses are done. Indeed, we have recently been able to show great promise for molecular restaging of stage 1 lung cancer using this approach and it is the combination of 4 genes, and particularly two of these p16 and H-cad (which is showing some promise, above, for breast cancer) are very robust **(*Brock, M.V., Hooker, C.M., Ota, E., Han, Y., Guo, M., Ames, S., Glöckner. S., Piantadosi, S., Gabrielson, E., Pridham, G., Pelosky, K., Belinsky, S.A., Yang, S.C., Baylin, S.B., Herman, J.G. Using DNA Methylation Markers to Predict Early Recurrence and to Re-Stage Patients with Stage 1 Lung Cancer. N Engl J Med 13;358:1118-1128, 2008).*** Key to these lung cancer results was the fact that ***we were able to analyze not only tumor DNA, but key staging lymph node DNA as well and the combination proved to be the ultimate key***.

Reportable Outcomes

The initial results upon which the Idea Award was based were published:
Douglas DB, Akiyama Y, Esteller, M, Gabrielson E, Weitzman S, Williams T, Herman JG, Baylin SB. Hypermethylation of a Small CpGuaine-Rich Region Correlated with Loss of Activator Protein-2α Expression during Progression of Breast Cancer. Cancer Research 64:1611-1620, 2004.

Conclusions

The status of the work is outlined in the Key Research Accomplishments and can only be called promising for further development at this stage. We will continue the work as outlined and the analyses. Most important, this work and access to the New Mexico cohort has spurred our continued efforts to add new genes to our panel. By a random screening procedure of breast cancer cell lines for DNA hypermethylated genes, extrapolation of the data to primary samples, and matching of DNA methylation data to database microexpression arrays, we have important results. We have found that a series of genes which have recently been shown to have low frequencies of mutations in breast and/or colon cancers, in genome re-sequencing efforts **(*Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, et al. The genomic landscapes of human breast and colorectal cancers. Science 318: 1108–1113, 2007*)** have a much higher incidence of DNA hypermethylation in breast cancer **(*Chan, T.A., Glockner, S., Yi, J.M., Chen, W., Van Neste, L., Cope, L. Herman, J.G., Velculescu, V., Schuebel, K.E., Ahuja, N., Baylin, S.B. Convergence of Mutation and Epigenetic Alterations Identifies Common Target Genes in Breast and Colon Cancer that Predict for Poor Clinical Prognosis. PLoS Med 5(5):e114, 2008*)**. Moreover, several of these genes (PTPRD, Syne1, COL7A1, EVL, and Ret) predict for poor survival in terms of low expression in the microarray databases. ***To continue all or our work in this proposal, and to add in the other genes, Dr. Ahuja who has helped with all of the above studies, Dr. Chan, the first author in the PLoS Med paper above and now at Memorial Sloan Kettering (MMSK), and Dr. Belinsky, our collaborator in New Mexico, have just received a joint Komen Foundation grant to continue study of the New Mexico cohort, and a MMSK cohort***. In summary, our original IDEA while not yet in any way proved to have robust value ahs also not been invalidated – and the studies we have begun are ongoing and will be played out over the next several years.

References

No papers have yet been submitted from the work done directly with support of this award.

Appendixes

None